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Letter

Anti-persister and Anti-biofilm Activity of Self-Assembled Antimicrobial Peptoid Ellipsoidal Micelles

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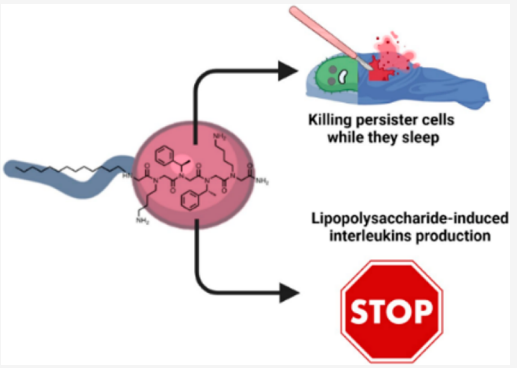
ABSTRACT: Although persister cells are the root cause of resistance development and relapse of chronic infections, more attention has been focused on developing antimicrobial agents against resistant bacterial strains than on developing anti-persister agents. Frustratingly, the global preclinical antibacterial pipeline does not include any anti-persister drug. Therefore, the central point of this work is to explore antimicrobial peptidomimetics called peptoids (sequence-specific oligo-*N*-substituted glycines) as a new class of anti-persister drugs. In this study, we demonstrate that one particular antimicrobial peptoid, the sequence-specific pentamer TM5, is active against planktonic persister cells and sterilizes biofilms formed by both Gram-negative and Gram-positive bacteria. Moreover, we demonstrate the potential of TM5 to inhibit cytokine production induced by lipopolysaccharides from Gram-negative bacteria. We anticipate that this work can pave the way to the development of new anti-persister agents based on antimicrobial peptoids of this class to simultaneously help address the crisis of bacterial resistance and reduce the occurrence of the relapse of chronic infections.



KEYWORDS: *peptoids, micelles, antibacterial, biofilm, persister cells*

Persister cells, a bacterial subpopulation of metabolically inactive (dormant) bacteria that display not antibiotic resistance but rather tolerance due to their dormancy,[1] cause relapse in chronic infections such as chronic suppurative otitis media (CSOM)[2] and cystic fibrosis.[3] Therefore, targeting persister cells, which constitute 1% of the biofilm population,[4] holds promise for truly eradicating biofilm-associated chronic infections. Conventional antibiotics such as fluoroquinolones, beta-lactams, and aminoglycosides target DNA replication, cell wall synthesis, and protein synthesis, respectively. These ATP-dependent targets are less active in dormant persister cells, which have low ATP levels compared to metabolically active bacteria.[5] Conventional antibiotics have also been shown to induce the persister cell phenotype, and the resulting sub-lethal DNA damage provides the grounds for development of antibiotic resistance to the original antibiotic and also cross resistance.[6] Therefore, conventional antibiotics are ineffective and potentially prolong the infection in persister-cell-associated infections. On the other hand, the targeting of persister cells can be achieved with drugs that have mechanisms of action that are independent of metabolic activity. For example, the DNA cross-linker cisplatin has been shown to eradicate persister cells.[7] However, the known toxicity of cisplatin in cancer patients and cisplatin-associated ototoxicity makes this anti-persister drug unsuitable for treating recalcitrant infections in CSOM.[8] Therefore, new anti-persister agents are needed for treating recalcitrant infections.

Antimicrobial peptides (AMPs), a key component of the innate immune system, are an attractive class of drugs to combat the emergence of multi-drug-resistant pathogens with their broad-spectrum activity, fast killing, and high cell selectivity. However, their potential as therapeutics is limited by high cost and short plasma half-life.[9] Antimicrobial peptidomimetics, such as peptoids, are designed to mimic the structure and activity of AMPs at shorter chain lengths, while demonstrating extreme stability to proteases compared to peptides. Additionally, peptoids are readily synthesized on a peptide synthesizer with reasonably priced, commercially available reagents and a stepwise sub-monomer process.[10] Recently, we showed that antimicrobial peptoids (including TM5) are active against all the *ESKAPE* pathogens, which are the primary cause of nosocomial (hospital-acquired) infections exhibiting virulence and multi-drug resistance,[11] as well as being active against both viruses[12] and fungi.[13] TM5 is a 5-mer

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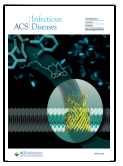

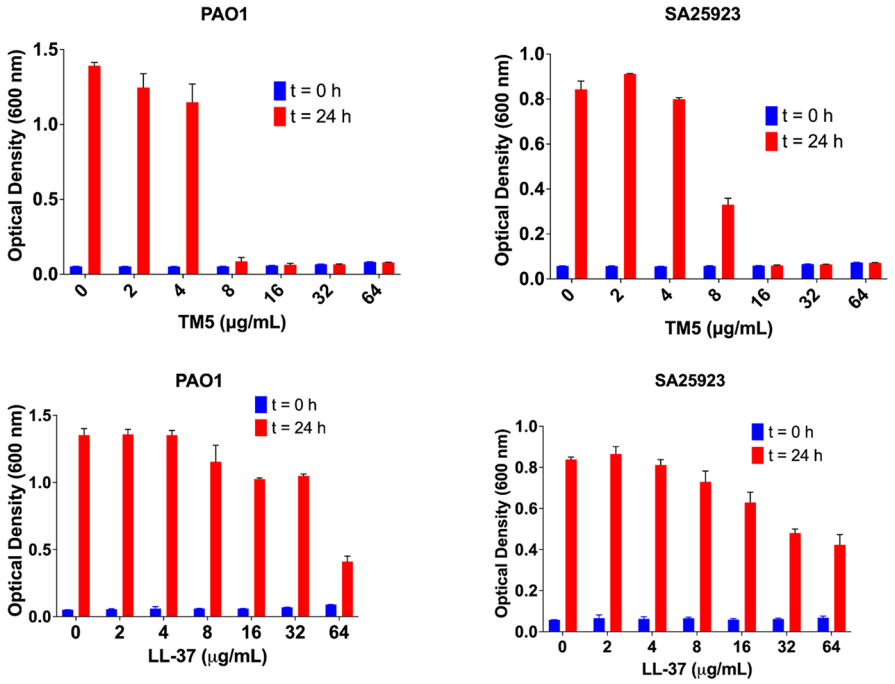

Figure 1. Comparison of the bactericidal activity of TM5 and LL-37 against those of *P. aeruginosa* (PAO1) and *S. aureus* (SA25923). OD_{600} is measured at t = 0 and t = 24 h. Minimal inhibitory concentration (MIC) is the lowest concentration that inhibits growth of bacteria after 24 h incubation at 37 °C. The data presented are the means ± SD of three independent experiments.

peptoid design with sequence H-*N*-tridec-*N*-Lys-*N*-spe-*N*-spe-*N*-Lys-NH_2.[13] The remarkably broad-spectrum activity of TM5 suggests that its mechanism of action is biomimetic; for instance, the human antimicrobial peptide LL-37 is also antibacterial, antifungal, and antiviral simultaneously.[14,15] Like all cationic AMPs and AMP mimics, peptoid TM5 can disrupt, depolarize, and cross anionic pathogen membranes. Mechanism of action studies published in 2017 also showed that this class of cationic, biomimetic antimicrobial peptoids can induce rapid aggregation of bacterial intracellular macromolecules, including DNA and ribosomes,[16] rigidifying the bacterial cytoplasm like LL-37.[17,18] Since this mechanism of action is independent of metabolic activity, we hypothesize that TM5 can be active against persister cells.

Although previous results support the use of peptoids to treat acute infections caused by metabolically active bacteria or the acute phase of chronic infections caused by slow-growing bacteria,[11] it was not clear whether peptoids can also serve as promising drugs to stop the relapse of chronic infections caused by metabolically inactive bacteria called persister cells. Furthermore, antibiotic failure in biofilm-based infections is due to persister cells because planktonic cells and biofilm of *Pseudomonas aeruginosa* exhibit similar resistance to killing by antimicrobials.[19] In support of this observation, we recently demonstrated that topical therapy failure in CSOM is due to the persister cells within biofilms.[2] Therefore, to stop the relapse of chronic infections with peptoids, it is important to also show activity against dormant persister cells in addition to metabolically active and slow-growing bacteria. Recently, the Cegelski lab noted that an antimicrobial agent can exhibit antimicrobial activity against persister cells in the presence of a carbon source (by awakening the persister cells) but lost its efficacy in phosphate buffered saline (PBS) in the absence of cell metabolism and growth (dormant persister cells).[20] The only paper that has investigated the bactericidal action of peptoids on persister cells demonstrated their activity on awakened persister cells (in the presence of a carbon source instead of PBS).[21] Therefore, the antimicrobial activity of peptoids against dormant persister cells remains an open question. In this study, we demonstrate that TM5 is active against planktonic persister cells and persister cells within biofilms. Moreover, we show that TM5 helps to decrease endotoxin-induced toxicity. Of note, we did not use crystal violet staining, which measures biofilm dispersion and prevention, or 2,3,5-triphenyl tetrazolium chloride (TTC), which measures surviving bacteria according to metabolic activity, to investigate the bactericidal activity of TM5 against biofilm. These methods are not suitable to demonstrate conclusively that antimicrobial agents are bactericidal against dormant persister cells. Eradication of persister cells can only be achieved by showing sterilization of biofilm because those cells only constitute 1% of the biofilm population.[4]

First, we examined peptoid self-assembly in aqueous solution using small-angle X-ray scattering (SAXS) and found that the peptoid self-assembles into well-defined core−shell ellipsoidal micelles (Figure S1) as a result of the amphiphilic properties of the molecule. Upon analysis of the data using a theoretical ellipsoidal core−shell micelle model,[11] the aggregation number was estimated to be 98, the larger core radius (R_{core}) of 20.8 Å, a smaller core radius (R_{core}) of 13 Å, and a thickness of shell (D) of 7 Å. The critical micelle concentration (CMC) was estimated to be approximately 1.4 μM in phosphate-buffered saline (PBS) buffer by measuring surface tension using the pendant drop method. The self-assembly of peptoids has previously been shown to correlate with high antimicrobial activity against metabolically active ESKAPE pathogens.[11] Furthermore, SAXS data confirms that TM5 mimics the self-assembly properties of AMPs such as the human cathelicidin, LL-37, which self-assembles into tetrameric bundles and therefore has a much lower aggregation number than TM5.[11]

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Since TM5 mimics the self-assembly ability of LL-37, we first compared the antimicrobial activity of TM5 to LL-37 by measuring the minimal inhibitory concentration (MIC). Concentrations of TM5 and LL-37 up to 64 μg/mL were tested, and optical density at 600 nm (OD_{600}) was measured at t = 0 h and t = 24 h after inoculation to determine inhibition of bacterial cell growth. As seen in Figure 1, the MIC values for TM5 against *Pseudomonas aeruginosa* (PAO1) and *Staphylococcus aureus* (SA25923) are 8 μg/mL and 16 μg/mL, respectively. In contrast, LL-37 fails to inhibit the bacterial growth at the highest concentration tested, which is in good agreement with earlier reported MIC > 100 μg/mL.[22,23] This data confirms the superior antimicrobial activity of TM5 over LL-37.

Having shown that TM5 is more effective than LL-37 against metabolically active bacteria, we next compared the potential of TM5 and LL-37 to eradicate 48 h biofilms from PAO1 and SA25923. To avoid awakening persister cells within the biofilm, all eradication treatments were performed in PBS. The biofilm on a Calgary Biofilm Device was treated at 37 °C and the drug was removed after 24 h (challenging plate). Then, the plate was washed and incubated in fresh Luria−Bertani (LB) broth at 37 °C for 48 h (recovery plate). In this method, the minimum biofilm eradication concentration (MBEC) is achieved when the optical density (OD_{650}) of the recovery plate at 650 nm is below 0.1.[24] As seen in Figure 2, the OD_{650} for PAO1 and SA25923 biofilms treated with TM5 at 70 μg/mL was less than 0.1. Moreover, to confirm sterilization of the biofilm after TM5 treatment, 48 h recovering phase was spotted on agar plates and incubated for an additional 48 h at 37 °C. No bacterial growth was observed after treatment with 70 μg/mL TM5, suggesting that this is the MBEC (Figure 2C). The biocidal activity of LL-37 was previously investigated either by quantifying reduction of biofilm biomass using a crystal violet assay[25] or by measuring log CFU (colony forming unitd) reduction.[22] A limitation of these studies is that they overlook persister cell survival after LL-37 treatment. For example, achieving an antimicrobial activity equivalent to 6-log reduction of the biofilm cells does not necessarily mean that persister cells were killed because they constitute only 1% of the biofilm population.[4] To demonstrate whether LL-37 can eradicate biofilm, we tested its bactericidal activity using the optical density (OD_{650}) of the recovery plate at 650 nm. Following treatment with LL-37 at 200 μg/mL, the OD_{650} for PAO1 and SA25923 biofilms was the same as the vehicle control (0 μg/mL; OD_{650} = 1). This data suggests that LL-37 fails to eradicate the biofilm at concentrations up to 200 μg/mL. Table 1 summarizes the MIC and MBEC values for TM5 and LL-37 for each bacterial strain.

To demonstrate that the TM5-induced biofilm eradication was due to its activity against persister cells, we evaluated the antimicrobial activity of TM5 against planktonic persister cells. A compound is active against bacteria if it shows a 3-log reduction in CFU/mL compared to the initial inoculum.[26] Of note, two different types of persister cells have been used in the literature to evaluate anti-persister activity of a potential drug. Type I persister cells are formed during the stationary growth phase while type II persister cells are obtained after treatment of the exponential growth phase with 4 × MIC of bactericidal antibiotics.[27,28] In contrast to type I, the antibiotic targets such as protein synthesis are still active in type II persister cells. Therefore, aminoglycosides can kill type II persister cells upon increasing the drug uptake.[29] Although the efficacy of peptoids against persister cells was recently reported, the peptoids were only tested against type II persister cells where conventional antibiotics are still effective.[21] To evaluate the potential of peptoids against type I persister cells formed during the stationary growth phase, we treated stationary phase cultures with ofloxacin at 5 μg/mL for 24 h as previously reported (Figure 3A).[30] In contrast to the previous protocol where type II persister cells were resuspended in growth medium,[21] our eradication treatments were performed in PBS to avoid awakening of the persister cells. As expected, TM5 treatment at 70 μg/mL showed a 3-log reduction in CFU/mL compared to the initial inoculum of isolated planktonic persister cells of PAO1 and SA25923 (Figure 3B).

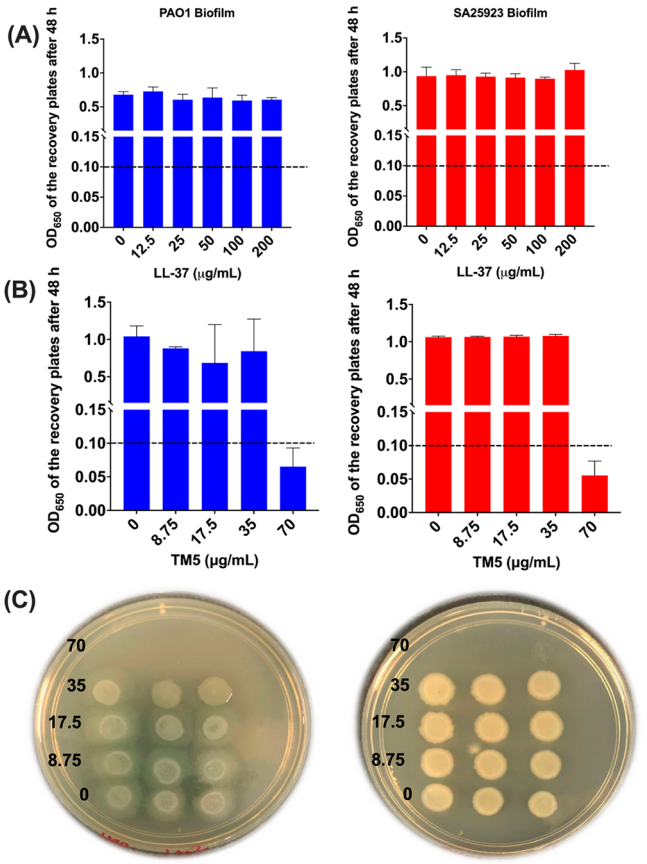

Figure 2. (A) Eradication of biofilm *P. aeruginosa* (PAO1) and *S. aureus* (SA25923) 48 h biofilms by LL-37 and (B) TM5. Following the treatment, the optical density (OD) from recovery plates after 48 h incubation was measured at 650 nm (OD_{650}) using a spectrophotometer. (C) Representative Petri dish showing eradication of biofilm following TM5 treatment. The number on the image represents TM5 concentration in μg/mL. The data presented are the means ± SD of three independent experiments.

Table 1. Susceptibility Profile of Planktonic Bacteria and Biofilms against TM5 and LL-37[a]

	TM5		LL-37	
bacteria strain	MIC (μg/mL)	MBEC (μg/mL)	MIC (μg/mL)	MBEC (μg/mL)
PAO1	8	70	>64	>200
SA25923	16	70	>64	>200

[a]MIC = minimal inhibitory concentration; MBEC = minimal biofilm eradication concentration.

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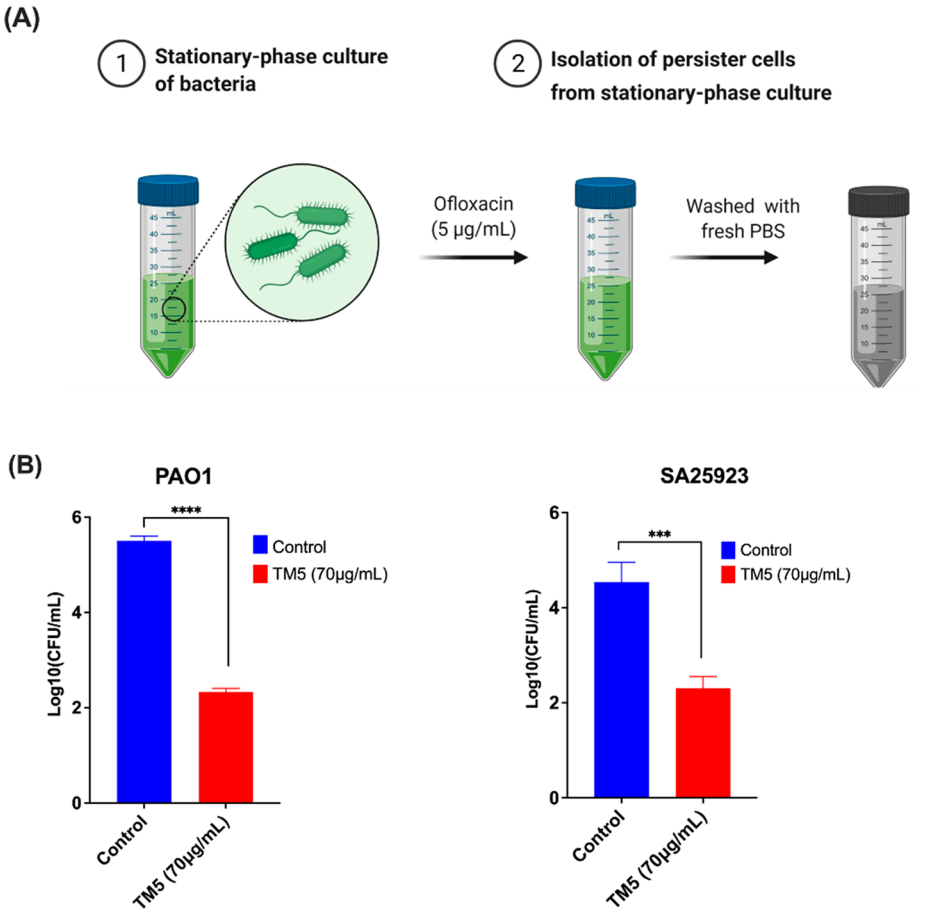

Figure 3. TM5 is active against planktonic persister cells of *P. aeruginosa* (PAO1) and *S. aureus* (SA25923). (A) Protocol showing the isolation of persister cells from stationary phase culture. (B) Survival persister cells after TM5 treatment was determined by viable plating (CFU/mL) and showed 3-log reduction compared to initial inoculum. The data presented are the means ± SD of three independent experiments.

Since it is well established that bacterial persistence promotes the evolution of antibiotic resistance,[6] anti-persister agents such as TM5 will not only stop recurrent infections but also benefit in the fight against antibiotic resistance. We performed a preliminary study of resistance development against TM5 by using *P. aeruginosa* (PA14), which along with PAO1, is one of two commonly used laboratory strains of *P. aeruginosa*. PA14 was grown overnight in the presence of TM5 (0.5 × MIC = 4 μg/mL). This process was repeated 30 times to evaluate the adaptive resistance development. No change in MIC of TM5 was observed after 30 successive exposures of PA14 to sub-MIC. In comparison, a 4-fold increase in MIC of gentamicin was observed using the same protocol. This data suggests that bacteria will be less prone to develop resistance against a potent anti-persister compound such as TM5 (Figure S2).

It is well known that hydrogen peroxide (H_2O_2) kills *S. aureus* by reacting with iron-mediated Fenton reaction to form hydroxyl radical.[31] We demonstrate that oxidative stress induced by H_2O_2 (3% = 0.98 M) sterilizes SA25923 persister cells (10^6 CFU/mL) while the same concentration of H_2O_2 failed to significantly reduce the PAO1 persister cell inoculum (10^6 CFU/mL) (Figure S3). As the interaction between TM5 and persister cells may induce oxidative stress, we used a reactive oxygen species (ROS)-sensitive probe, dichlorofluorescein, to quantify TM5-induced oxidative stress. The fluorescence intensity of the ROS-sensitive probe (area under the curve) was enhanced 7-fold in PAO1 persister cells and 3-fold in SA25923 persister cells treated with TM5 (70 μg/mL) compared to untreated controls (Figure S4). Based on these results, we hypothesize that anti-persister cell activity of TM5 may involve a combination of oxidative stress and aggregation of bacterial intracellular contents as previously reported.[16] Moreover, this non-specific mode of action gives TM5 a significant advantage compared to conventional antibiotics to simultaneously combat persister cells and bacterial resistance development.

Having shown the anti-persister activity of TM5, we further demonstrated its potential benefit in reducing the local inflammatory response, which is secondarily damaging, and is partly driven by interleukin-6 (IL-6) and interleukin-8 (IL-8). We first determined the cytotoxicity of TM5 against A549 lung epithelial cells to identify a non-cytotoxic concentration. A549 cell exposure to 50 μg/mL of TM5 for 24 h exposure did not cause cytotoxicity (cell viability >80%) (Figure 4A). The TM5 concentration that induces 50% of cell death (IC_{50}) is 106.7 μg/mL (Figure 4B). Up to at least 256 μg/mL, TM5 has been shown to be safe on 3D human primary cultures of epithelial cells[11,12] grown at the air−liquid interface, which is also supported by the TM5 *in vivo* biosafety profile observed with topical treatment in a *S. aureus* murine incision wound model.[32]

Next, we evaluated the IL-6 and IL-8 levels produced by A549 cells upon exposure to *P. aeruginosa* lipopolysaccharide

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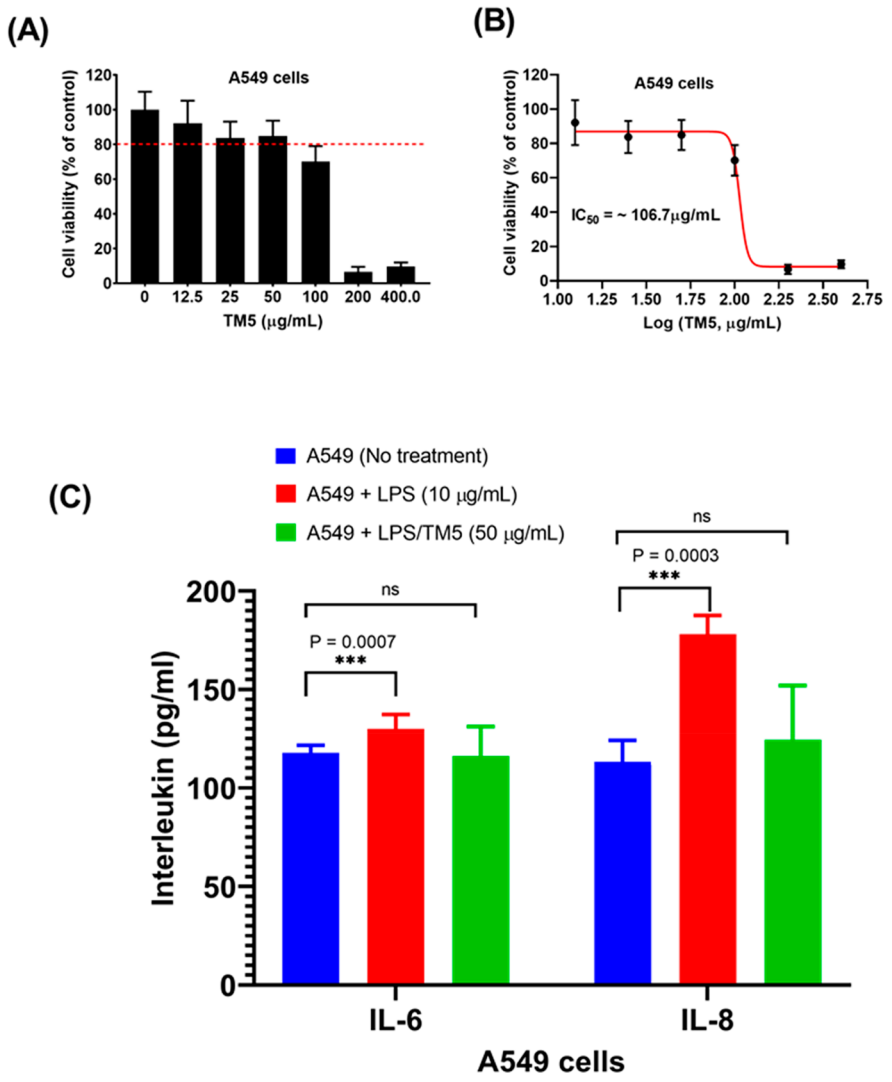

Figure 4. TM5 inhibits PAO1 LPS-induced cytokine production by A549 cells. (A) Concentration-dependent cytotoxicity of TM5 against A549. (B) Determination of IC50. A non-cytotoxic concentration of TM5 (50 μg/mL) reduces levels of LPS-induced IL-6 and IL-8 secretion in A549. The data presented are the means ± SD of three independent experiments.

(LPS) (10 μg/mL). We found that the cytokine levels were significantly increased compared to untreated controls, while co-treatment of LPS and TM5 (50 μg/mL) showed no significant difference in the IL-6 and IL-8 levels compared to untreated controls (Figure 4C). We conclude that TM5 inhibits LPS-induced cytokine production by A549 cells probably due to complex formation between LPS and TM5. This data supports the further exploration of TM5 as a treatment to mitigate the local inflammatory response in response to *P. aeruginosa* infection.

In summary, this paper tests the anti-persister activity of TM5, which can self-assemble into well-defined core−shell ellipsoidal micelles. We have demonstrated TM5 is a multifunctional platform that can be used to eradicate biofilms and mitigate LPS-induced cytokine production. Future studies will explore the *in vivo* anti-persister cell activity of TM5 using a chronic biofilm infection animal model. Furthermore, the antimicrobial molecular mechanisms of TM5 seems to prevent resistance development. Although our resistance study showed that resistance development against TM5 is unlikely, further studies need to be performed to determine the potential resistance mechanisms.

METHODS

Peptoid Synthesis. Peptoids were synthesized manually in accordance with the submonomer method.[10] The detailed procedure can be found in the Supporting Information. UPLC chromatograms are shown in Figure S5.

Small Angle X-ray Scattering (SAXS). This technique allows for the determination of whether biomacromolecules like peptoids self-assemble into nanostructures or instead exist as single molecules in aqueous solution.[33−35] The morphology of the structure can be deduced through detailed theoretical modeling. The procedure can be found in the Supporting Information.

Critical Micellar Concentration (CMC) Measurements. The CMC of TM5 in PBS was determined using the pendant drop method on an OneAttension Theta optical tensiometer (Biolin Scientific). A ~10 μL drop containing the peptoid was suspended from a 1 mL gastight Hamilton syringe fitted with an 18-gauge blunt needle. Images of the droplet were collected using a Gigabit Ethernet camera (76 frames/s at 782×582 resolution). The drop shape was fit to the Laplace−Young equation using OneAttension software to obtain the shape

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factor and the surface tension. The surface tension was then plotted against the log concentration of peptoid and the CMC determined as the intersect between the regression straight line of the linearly dependent region and the straight line passing through the plateau (Figure S6).

Bacterial Strains and Culture Conditions. The laboratory strains of *P. aeruginosa* PAO1 and PA14 and *S. aureus* SA25923 were purchased from ATCC. All organisms were cultured in Mueller Hinton broth (MHB) from individual colonies at 37 °C, shaking at 200 rpm.

Minimum Inhibitory Concentration (MIC). The MIC of TM5 and LL-37 were determined against PAO1 and SA25923 planktonic strains using the broth microdilution method.[36] The bacteria were grown overnight at 37 °C in LB medium. Subsequently, the drug was mixed with bacterial inoculum in MHB (optical density OD at 600 (OD_{600}) <0.1) using the serial dilution method (2-fold) in a 96-well polypropylene microplate and incubated at 37 °C for 24 h. Next, the bacteria growth measured by the optical density change was evaluated at OD_{600} in a microplate reader (SpectraMax M2, Molecular Devices, Downington, PA). The MIC was determined as the concentration where there is no significant change in the OD_{600} between the initial (t = 0 h) and final (t = 24 h) reading. Experiments were performed in biological triplicates.

Determination of Minimum Biofilm Eradication Concentration (MBEC). Overnight cultures of PAO1 and SA25923 were diluted 1:1000 in fresh MHB medium, and 150 μL of the dilution was added per well in an MBEC Assay Biofilm Inoculator with 96 wells. Biofilms were allowed to develop onto peg lids for 48 h without shaking. The peg lids were gently rinsed to remove planktonic bacteria and incubated in a new MBEC Assay Biofilm Inoculator with 96 wells containing a serial dilution (2-fold) of tested drugs in PBS for 24 h. Then the peg lids were washed and placed in a new MBEC Assay Biofilm Inoculator with 96 wells containing fresh medium (recovery media). Following incubation for 48 h, the optical density at 650 nm (OD_{650}) was read using the microplate reader. The MBEC value was defined as the lowest drug concentration that eradicates the biofilm (OD_{650} > 0.1). To confirm the MBEC, after the additional 48 h incubation, the entire 200 μL volume of the well is centrifuged in a microcentrifuge tube, the supernatant was removed, the entire pellet resuspended in 5 μL, and this suspension spotted on a plate to ensure that all of the bacteria is on the agar plate and no bacteria remains at the bottom of the well. The LB agar plates were then incubated at 37 °C without shaking for 48 h. Three independent experiments and three replicates were performed.

Persister Cell Isolation. Persister cells of bacteria PAO1 and SA25923 were selected by exposing overnight stationary phase cultures to ofloxacin at 5 × MIC (5 μg/mL). The bacteria were incubated with the drug for 24 h at 37 °C under shaking at 200 rpm. Following this step, the samples were washed with PBS and serially diluted to determine the colony-forming unit per milliliter (CFU/mL). Exposure of the bacteria to this concentration level will eradicate all metabolically active bacteria; the remaining population of bacteria being persister cells.

Persister Cell Killing. Isolated persister cells from PAO1 and SA25923 were treated with tested drugs in PBS for 24 h at 37 °C under shaking at 200 rpm. Second, the bacteria cells were washed three times with PBS and serially diluted to determine the colony-forming units per milliliter (CFU/mL). Three independent experiments and three replicates were performed.

Cell Culture and *In Vitro* Cytotoxicity Assay. The human lung adenocarcinoma cell line (A549) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. The cells were incubated in 5% CO_2 humidified at 37 °C for growth. The cytotoxicity induced by TM5 was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A549 cells (2×10^4/mL, 100 μL/well) were seeded in 96-well plates. After 24 h, the cell culture was exposed to concentrations of TM5 ranging from 0 μg/mL to 400 μg/mL (TM5 was dispersed in DMEM). After 24 h of incubation, the medium containing TM5 was removed, and cells were washed with PBS and incubated with fresh cell culture medium for another 24 h. Then, 20 μL of the MTT (5 mg/mL) was added to each well, followed by incubation for 4 h in 5% CO_2 humidified at 37 °C. The medium was removed carefully, and 200 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve formazan crystals. The absorbance of formazan was read at 595 nm using the microplate reader. A blank solution (0 μg/mL of TM5) was tested, and no cytotoxicity could be observed. Three independent experiments and three replicates were performed. Results were analyzed as the average viability (% of the untreated control) ± standard deviation (SD).

Cytokine Measurement. The cytokine measurements were performed using human IL-6 and IL-8 ELISA kits according to manufacturer's protocols (R&D Systems, Inc., Minneapolis, MN). Three independent experiments and three replicates were performed.

Reactive Oxygen Species (ROS) Determination. Persister cells of PAO1 and SA25923 were grown, washed, and resuspended in PBS. The cell suspension was treated with TM5 for 1 h at 37 °C, 180 rpm. Next, 5 μM 2′,7′-dichlorofluorescin diacetate (DCFDA) was added to the cell suspension, which was then incubated in the dark at 37 °C for 30 min. The fluorescence was measured using a microplate reader with excitation and emission wavelengths set at 488 and 530 nm, respectively. Three independent experiments and three replicates were performed.

Statistical Analysis. Statistics were performed using GraphPad Prism 9.0. Data was tested using an unpaired (independent) t test in GraphPad Prism to show which groups are significantly different from each other. Data are presented as mean ± SD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.2c00288.

Detailed synthesis and purification of peptoids; detailed experimental procedure and results from SAXS; change in TM5 MIC after 30 successive exposures of *P. aeruginosa* (PA14) to TM5 at 0.5 × MIC (4 μg/mL); bactericidal action of hydrogen peroxide; production of reactive oxygen species by TM5; and UPLC chromatogram of TM5, including Figures S1−S6 (PDF)

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Author Contributions

J.S.L. and L.A.B. contributed equally.

Notes

The authors declare the following competing financial interest(s): A.E.B. is a shareholder and member of the Board of Directors of Maxwell Biosciences; N.M., G.D., and H.J. are shareholders and consultants for Maxwell Biosciences.

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